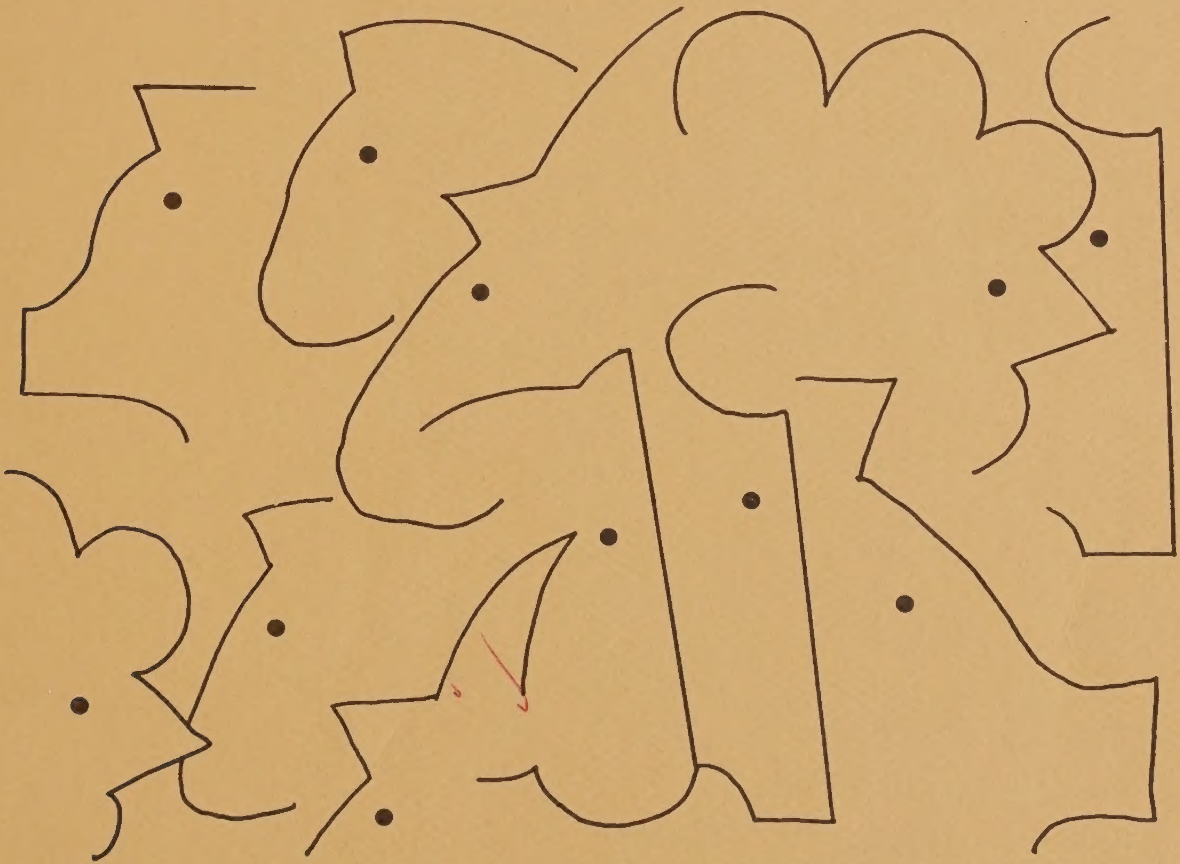
Diagnosis of African Swine Fever
(Fluorescent Test)

PLUM ISLAND ANIMAL DISEASE CENTER



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THE FLUORESCENT ANTIBODY (FA) TEST FOR AFRICAN SWINE FEVER (PIADL METHOD)

Method outlined by: Dr. I. C. Pan
Procedures used by the Diagnostic
Investigations Division, PIADL

1. Preparation of fluorescein-conjugated antibody.

1.1 Antiserum to ASF Virus

A high antibody titer in terms of precipitating antibody is a prerequisite for obtaining a high quality fluorescein conjugate. (Note: this conjugation method has been used for sera of swine, rabbits, and mink.)

1.2 Preparation of crude gamma-globulin from the antiserum.

A crude gamma-globulin fraction can be obtained by mixing a half volume of saturated ammonium sulfate with a

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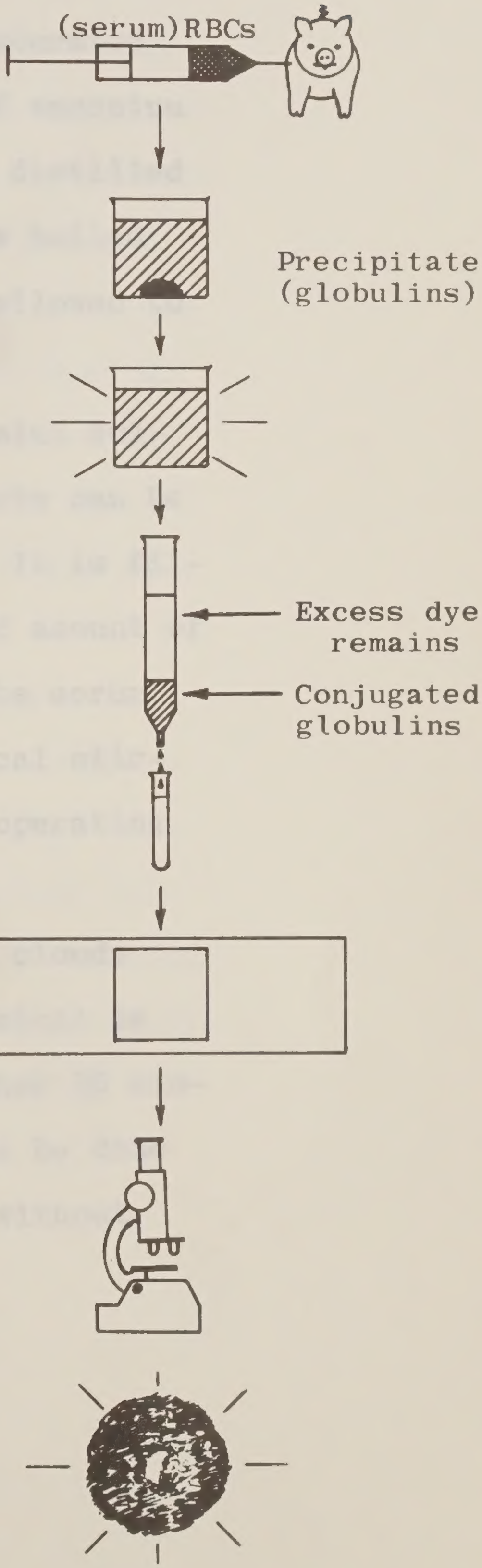
BASIC STEPS IN THE FLUORESCENT ANTIBODY (FA) TEST FOR AFRICAN SWINE FEVER (ASF) ANTIGENS

1. Serum containing antibodies of high titer obtained from swine infected with ASF virus.
2. Globulins in serum precipitated with ammonium sulfate.
3. Precipitate (freed from ammonium sulfate) reconstituted in buffer solution; conjugated to fluorescein dye.
4. Excess dye removed by column chromatography.
5. Conjugated antiserum added to smear or frozen section on microscope slide.
6. Section examined under ultraviolet (UV) light microscope.
7. Cells containing ASF antigen fluoresce under UV light.



volume of serum. To prepare saturated ammonium sulfate, about 1 kg of ammonium sulfate is added to 500 ml. of distilled water (D H_2O). This mixture is boiled for about 20 minutes and then allowed to cool to room temperature.

After the undissolved ammonium sulfate sediments out, the supernate can be used as a saturated solution. It is filtered before use. The required amount of ammonium sulfate is added to the serum dropwise with adequate mechanical stirring (a magnetic stirring bar operating at a slow speed).

The mixture should be very cloudy after the last drop of the chemical is added. It is stirred for another 20 minutes. The entire operation can be done at room temperature (22-28°C) without impairing antibody activity.

The mixture is then centrifuged at a low speed (1,900 X G) for 30 minutes at 6°C. At PIADL an International Equipment Company (AEC), Needham, Mass., USA, Model PRJ centrifuge having an 8 inch radius, when measured to a depth of 1/2 the liquid in the centrifuge tube, was used at an average speed of 3,000 r.p.m. at 6°C for this operation. (Appendix E has a graph which may be used for converting r.c.f. to r.p.m.)

After decanting the supernate the pellet is retained; this contains gamma globulin (antibody). Enough D H_2O is added to reconstitute the original volume of serum. The pellet should contain enough concentration of ammonium sulfate salt to dissolve the gamma globulin after addition of the D H_2O.

The process of washing is repeated. The pellet is resuspended in 1/3 saturated ammonium sulfate solution and centrifuged again. This process is repeated until the last pellet is completely white. This pellet is dissolved in the smallest amount of 0.01 M Tris-HCl buffer (pH 9.0) possible. To prepare the buffer, 12.1 gm of Tris (hydroxymethyl) aminomethane is dissolved in 1,000 ml. of D H_2O. The pH is adjusted with a pH meter to 9.0 by adding HCl drop wise. After the gamma-globulin is dissolved in the Tris-HCl buffer, the solution should have a bluish opalescent appearance. It is centrifuged at about 1,100 G for 30 minutes (see Appendix E to convert r.c.f. to r.p.m.) The supernate is collected for the next step.

1.3 Removal of contaminated ammonium sulfate.

The gamma-globulin solution is poured into a dialyzing tube of small caliber in order to hasten the dialyzing process and dialyzed against a large quantity of the same 0.01 M Tris-HCl buffer overnight, using a magnetic stirrer in the cold (5°C). The volume of dialyzing solution required will be more than 200 times the volume of gamma-globulin prepared. The next frames illustrate the method of preparing and utilizing dialysis tubes.

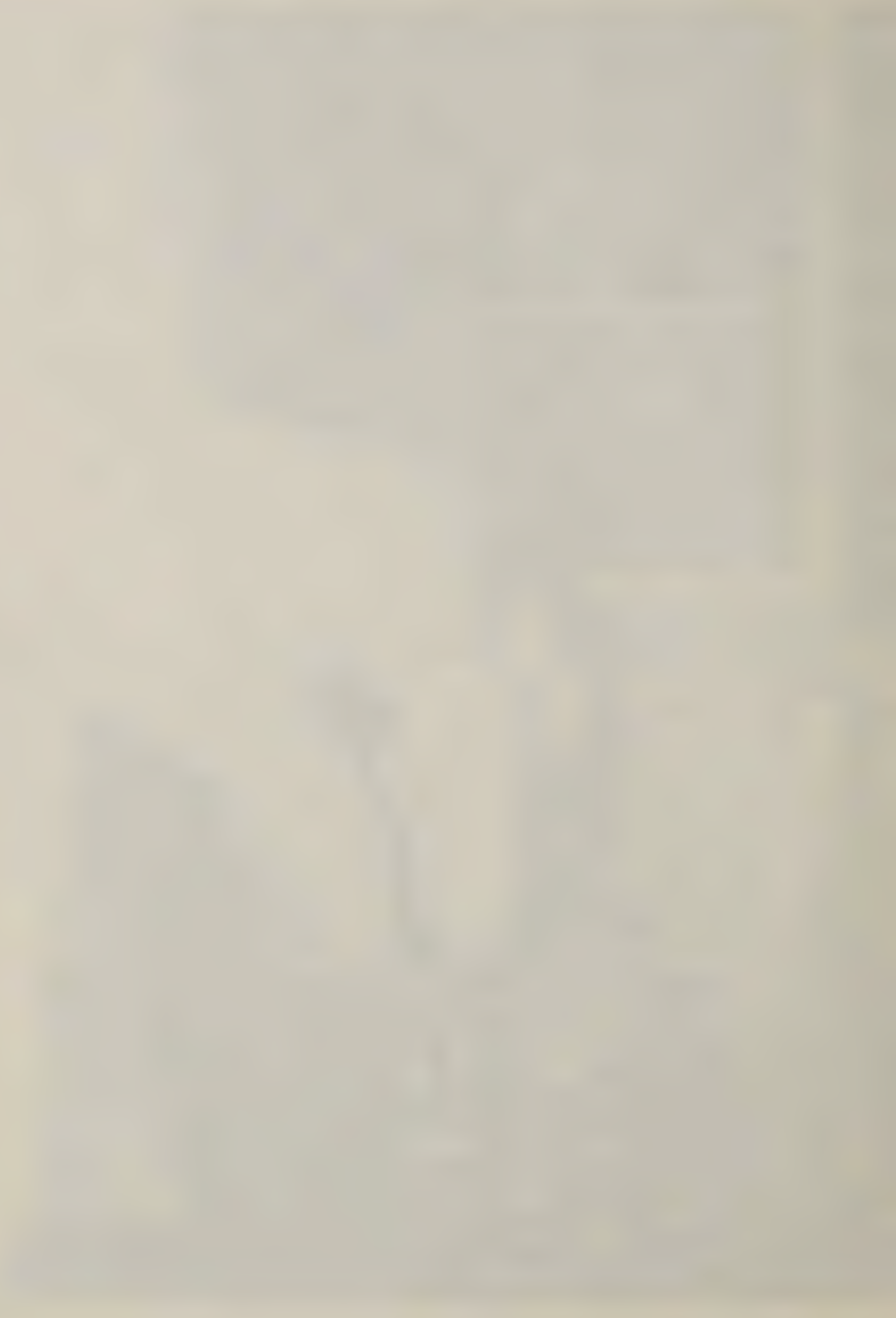
As an alternative method, a column of Sephadex G-25 (fine), suspended in and equilibrated with 0.01 M Tris-HCl buffer can be used in removing ammonium sulfate. A typical column used for this purpose is shown in Frame B-12. (A technique of equilibrating Sephadex in buffer is given in detail later.) The gamma-globulin prepared by this method, however, will be diluted 2 to 4 times and therefore will require concentration of the eluate before conjugation with fluorescein isothiocyanate. Concentration may be accomplished by pervaporation in dialysis membranes (A-7 to A-9), Diaflow filtration (Amicon Corporation, 280 Binney Street, Cambridge, Mass., USA), negative pressure dialysis, dialysis against powdered polyvinylpyralidone, Sephadex G-200, or similar means.

2. Conjugation of fluorescein isothiocyanate to antibody.

2.1 After the gamma-globulin is freed from ammonium sulfate, the total protein content is measured by the biuret or other appropriate method.

To 0.1 ml of sample is added 0.9 ml of PBS[2]. Five ml of the biuret reagent is added to each tube containing gamma-globulin. The sample is transferred to a quartz spectrophotometer cuvette (B-7). After 30 minutes at room temperature (21 to 23°C), the amount of protein is determined by means

[*] Biuret Reagent, Gornall, Bardawill and David (B. & L. No. 12) Fisher Scientific Company, Fair Lawn, New Jersey, USA.



of a spectrophotometer** (B-2) reading at a wave length of 505 nm. Standard serum of known protein concentration was treated the same way. One blank, containing 1 ml PBS[2] and 5 ml biuret reagent was also measured in the same manner. The optical densities of the test sample, control and standard are read. Protein concentrations are calculated as follows:

$$\frac{\text{O.D. of Test Sample}}{\text{O.D. of Standard}} \text{ X Protein concentration of standard} = \text{Protein concentration of test serum.}$$

**The instrument used is a Model DB-G Grating Spectrophotometer, Beckman Instruments, Inc., South Pasadena, California, USA.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2.2 The ratio of the gamma-globulin to the fluorescein dye should be 100:1 by weight. Therefore, to a total protein (gamma-globulin or other sample) content of 1,000 mg, 10 mg of fluorescein dye will be added.

Comparative tests should be made of different commercial fluorescein dyes to determine the most appropriate proportion of protein and dye. Fluorescein Isothiocyanate, Isomer I (from the Sigma Chemical Company, 3500 DeKalb Street, St. Louis, Mo. 63118, USA) has been found to be quite satisfactory.

Conjugation should be carried out at cold room temperatures (about 5°C). The gamma-globulin to be conjugated is placed in a beaker of appropriate size with a magnetic stirring rod and the beaker placed on a magnetic stirrer.

The magnet is operated at a speed which will not create bubbles, since these will denature protein. The premeasured fluorescein dye is placed on top of the gammaglobulin solution with magnetic stirring; it will first float on the surface, but then gradually dissolve into the liquid. The conjugation process is continued overnight at 5°C.

2.3 Removal of unconjugated fluorescein dye

A glass column of 5 cm in diameter by 60 cm in height is packed with Sephadex G-25 and equilibrated with a 0.01 M phosphate buffered saline (PBS[2]).

2.4 Preparation of PBS[2] (0.14 M NaCl):

The 0.1 M stock solution (pH 7.5) is prepared as follows:

1) KH_2PO_4 (Molecular weight = 136.091) 6.8 gm
2) NaH_2PO_4 (Molecular weight = 141.960) 41.18 gm

are dissolved in

3) 300 ml of hot D H_2O
4) Add 3,400 ml of D H_2O to complete the 0.1 M stock solution.

To complete the 0.01 M PBS[2], dilute one volume of the above with 9 volumes D H_2O and add 8.2 gm NaCl per L. If a pH of 7.3 is not achieved at once, it may be adjusted by drop-wise addition of concentrated phosphoric acid or 1 N NaOH as needed. (Appendix G has a formula for preparing NaOH.)

The 0.01 M PBS^2 may also be made directly by mixing the above chemicals with total volume of D H O while monitoring with a pH meter. The pH usually drops to about 7.3 when the NaCl (8.2 gm) is added. Adjustment is done as described.

2.5 Loading and eluting the conjugated protein in the column is done with this PBS^2 + NaCl. The conjugated protein will descend in the Sephadex gel as a yellowish-green band. The unconjugated free dye will be trapped at the top of the column.

The conjugated protein is collected by a mechanical fraction collector, or simply in a beaker. Because of its distinctive yellow-green color, it is not difficult to collect the desired fraction manually.

3. Removal of nonspecific fluorescence by DEAE-cellulose column chromatography.

3.1 The native gamma-globulin has almost negligible electric charges on its molecular surfaces at a pH of 7.3. However, after fluorescein dye (with a negative charge) is conjugated to the globulin, the latter acquires new negative charges on its surface. Since the binding of the dye to the protein is in random fashion, some protein molecules acquire more fluorescein dye than others. More fluorescein on the protein molecule means more negative charges on it; the protein will therefore bind to any substance of the opposite charge, which is the cause of certain nonspecific staining. Those groups of protein molecules which have acquired excessive negative charges can be removed by DEAE-cellulose chromatography.

3.2 DEAE-cellulose is preferred to DEAE-Sephadex. If a DEAE-cellulose of 1 milliequivalent is used, the ion-exchange capacity is almost the same as that of DEAE-Sephadex of higher milliequivalence in a given column. Furthermore, the cellulose is easier to pack. Before the DEAE-cellulose is packed in the column, it must be regenerated by being alternately treated with alkali and acid. To do this, the DEAE-cellulose is suspended in a large volume of distilled water. It is allowed to stand about 10 to 20 minutes and the supernate decanted. The cellulose is resuspended in distilled water.

These operations are repeated at least 2 or 3 times. This process will remove the "fines," fine particles of cellulose which would otherwise clog the

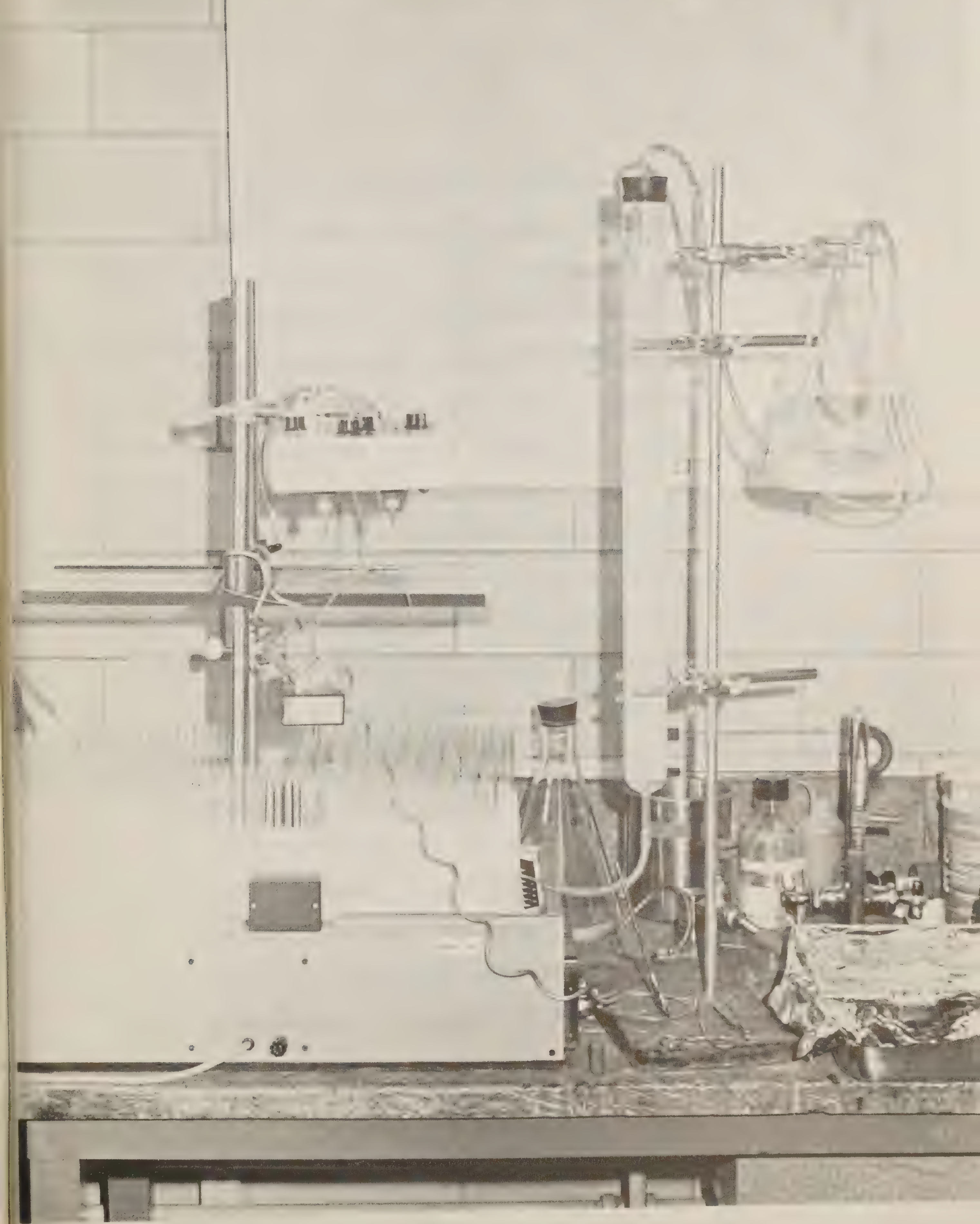
column and interfere with the flow rate of the eluate.

3.3 The water is next removed from the cellulose by a Buchner funnel. The resulting dry cake of cellulose in the Buchner funnel is then suspended in a large quantity of 0.5 M NaOH (20 gm per L D H_2O) in a 1 M NaCl (58.46 gm per L D H_2O) solution. This mixture is stirred for about 10 minutes and the cellulose again reduced to a dry cake by means of the Buchner funnel. This cake is then suspended in a 1 M NaCl solution and the NaOH removed by means of the Buchner funnel. The process is repeated until the effluent from the Buchner funnel is nearly neutral.

The cake is then resuspended in 0.5 N HCl in a 1 M NaCl solution. To prepare this solution mix 50 ml of

concentrated HCl and 950 ml of a 1 M NaCl solution. Agitate the cellulose in this acid solution for 10 minutes. The cellulose is then reduced to a cake with the Buchner funnel and the washing with 1 M NaCl is done in the same manner as with the alkali solution. When the effluent becomes neutral, or nearly so, the alkali treatment is repeated. When the effluent is again nearly neutral, the cake is packed in the Buchner funnel.

3.4 Suspend this cake in PBS^2 at pH 7.3 and continue to wash the cellulose by means of the Buchner funnel and repeated changes of buffer solution until the effluent from the washings shows the same pH and electric conductivity as the PBS^2. (The same PBS^2 may be used with DEAE-Sephadex.)



3.5 The cellulose suspension is now ready to pack in the column. The same size column as is used in Sephadex gel filtration is satisfactory. It may be found convenient to pack the regenerated DEAE-cellulose in the column by siphon. Any dissolved gas is removed by vacuum before the column is packed.

3.6 After the column is packed, a piece of filter paper cut to the size of the inside diameter of the column is placed on top of the packed cellulose. At least 3 column volumes of PBS[2] should be allowed to flow through the column to be sure that it is equilibrated. The head of fluid above the filter paper is reduced to about 2 mm, and the column is loaded with the conjugated protein in such a way that the cellulose is not disturbed. One method is to add the

conjugated protein by pipette to the inside of the glass column above the fluid. After the protein has entered the column it is eluted by PBS[2] at the proper flow rate. When the conjugate in the column nears the orifice at the bottom, the effluent is collected.

The strength of the conjugate must be determined in actual staining trials. After properly adjusting the antibody concentration, Sodium Azide at the final concentration of 0.1% is added as an antibacterial agent. The conjugate is then filtered through a 0.22 mu Millipore* filter, distributed in small quantities in vials and stored in a deep freezer at -70°C.

*Millipore Corporation, Bedford, Mass., 01730, USA.

4. Staining

Frozen sections and stamp (touch) smears.

4.1 Frozen sections are cut from 4 to 8 μ thick in a Cryostat. The sections are adhered to glass slides and dried on a hot plate at about 40 to 45°C for quick drying. A hair dryer with a hand grip is very useful also. Touch or stamp smears are prepared by touching the surface of the tissue to a clean glass slide and drying in air.

As soon as frozen sections (or smears) are dry, they are fixed in a dry acetone for 3 minutes at room temperature. If cell cultures are to be stained, it is recommended that they also be fixed in such acetone (precooled to -20°C in a freezer beforehand) for 30 minutes at -20°C. This procedure helps to avoid autofluorescence.

Specimens are transferred to PBS[2] at pH 7.3 and agitated for about 2 minutes. Next, a drop or 2 of fluorescein conjugate is spread over the specimen and allowed to remain 30 minutes at room temperature. To avoid drying, filter paper moistened with PBS[2] is placed in the staining chamber below the slides.

After staining, specimens are washed with 3 changes of PBS[2], 3 minutes apart, using frequent agitation. To process a large number of specimens, slides are placed in a staining rack and washed for 20 minutes in a vessel large enough to permit use of a magnetic stirrer. After washing, specimens are mounted in 90% glycerol in PBS[2]. Cell cultures on coverslips should be dipped in distilled water before mounting with glycerol. The washing in distilled

water removes salts which will interfere with examination by fluorescent microscopy.

Comment:

Although the conjugated antibody prepared by this method does avoid much nonspecific staining, some special situations warrant the adsorption of the final product with acetone dried guinea pig liver powder or similar material. This is particularly necessary when the antiserum contains antibody against Forsman antigen (either natural or acquired through immunization with Forsman antigen in a naturally negative animal, such as the rabbit). If it is desired to remove the antibody, one must absorb Forsman antibody with Forsman antigen (guinea pig liver or kidney powder). Otherwise, the conjugate does not

require further treatment.

The next frame is a photomicrograph (reproduced from a 35mm color slide) showing positive immunofluorescence for ASF in swine tissues examined by ultraviolet light microscopy. The slide was prepared as a stamp or touch smear. The significant immunofluorescence is in the central mass, a tissue cell containing specific ASF virus or antigen. The other less brightly fluorescing particles may be fragments of cells containing antigen or they may represent nonspecific fluorescence. Experience is required to properly interpret this test.